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An unexpected methyl group migration during on-column Stille derivatization of RNA

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ABSTRACT

Postsynthetic Stille cross-coupling for functionalization of oligonucleotides on solid support was applied on iodo modified RNA utilizing different protecting group strategies. As result, the otherwise very successful ACE [bis(acetoxyethyloxy)-methyl orthoester] chemistry was found to be limited since methylated side-products formed as was investigated via enzymatic degradation of RNA and various monomer model reactions. Enzymatic digestion of poly uridine sequences revealed presence of considerable amounts of N^3-methylated uridine derivatives due to migration of methyl as phosphate protecting group used in ACE strategy. Monomer test reactions mimicking conditions on RNA clearly indicated an enhanced methylation effect correlated to the Stille coupling procedure.

1. Introduction

Modified oligonucleotides, and in particular, labeled RNA, represent valuable tools in biochemical and medicinal research and are extensively applied for analytical and therapeutic purposes.[1–3] Hence, there is a growing demand in synthesis of oligonucleotides and efficient introduction of specific labels to obtain desired functionalized nucleic acid compounds.[4,5]

In general, chemical derivatization of synthetic oligonucleotides can be achieved via insertion of modified phosphoramidites during solid phase synthesis or by postsynthetic approaches.[5] The last-mentioned concept is non-linear and profits from greater flexibility in regard to using different labels eventually. There are several reports on postsynthetic methods on DNA, e.g., reviewed by Marx et al.,[4] and also few on RNA, recently discussed by us.[6]

Obviously, an effective automated RNA synthesis in high yields is a general prerequisite for postsynthetic derivatization. It is a widely accepted fact that RNA synthesis is more challenging compared to DNA synthesis—lower coupling efficiency in the synthesis cycle as well as the necessity to permanently block the 2′-hydroxyl function complicates the setup for RNA.[7–9] The most commonly used protecting group strategy consists of the 'classical' TBDMS[10,11] (*tert*-butyldimethylsilyl) approach, which can also involve alternative utilization of 2′-*O*-TOM[12,13] (triisopropylsilyloxymethyl) groups resulting in reduced steric hindrance, and thus, in increased coupling rates. This, as well as the emergence of ACE[14,15] chemistry in the first instance,[5,16] and of lately developed TC[17] (1,1-dioxo-1λ^6-thiomorpholine-4-carbothioate) strategy, too, are described to improve the outcome in RNA synthesis. Actually, these advances are reported to render automated RNA synthesis almost as reliable as DNA synthesis.[14,17]

Selection of the labeling reaction for postsynthetic derivatization on-column, which benefits from advantages in solid phase synthesis, such as high yields and ease in separation and purification, is dependent on compatibility with RNA stability, solid support conditions and chosen protecting group chemistry.

Our group is interested in postsynthetic palladium-catalyzed reactions on oligonucleotides in general. While Sonogashira cross-couplings on-column on DNA have been first reported by Grinstaff and Khan,[18] followed by Wagenknecht et al.[19] and us,[20–22] we pioneered the application also on RNA[22–26] employing TBDMS as well as ACE protection strategies.

Recently, we further expanded our on column concept for postsynthetic derivatization on RNA to Stille cross-coupling and could show the versatility of our new method.[6] Therein, functionalization by Stille reactions was performed on iodo modified

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precursor RNA oligonucleotides, which were synthesized via TBDMS, ACE[14] and TC[17] protecting group strategies, and transformation of the iodo function (in terms of complete conversion) to attach the desired label was found to be successful independently of applied chemistry.

However, remarkable differences in exclusive product formation, yield, purity, and efficiency could be observed, and herein results particularly referring to ACE chemistry are reported in more detail.

2. Results and discussion

2.1. Stille cross-couplings on RNA synthesized via TBDMS, TC, and ACE chemistry

Most experiments were carried out with commonly used TBDMS-protected RNA (**ON 1**, compare Table 1) that is bound to cpg (controlled pore glass) solid phase material—this turned out to be fully compatible with conditions of Stille cross-coupling, and completely converted RNA was isolated in yields comparable to unmodified oligonucleotides.[6]

Table 1
RNA oligonucleotides discussed within this manuscript[a]

Name	Sequence	Comment
ON 1	5′-UUU UUU U*UU UU	Via TBDMS chemistry, furyl-modified
ON 2	5′-UUU UUU U*UU UUU	Via TC chemistry, furyl-modified
ON 3	5′-UUU UUU U*UU UUU	Via ACE chemistry, furyl-modified
ON 4	5′-UUU UUU UUU UUU	Unmodified, for control purposes
ON 5[b]	5′-UUU UUU U*UU UUU	Via ACE chemistry, furyl-modified

[a] Synthesis, postsynthetic derivatization by Stille coupling, purification and characterization of **ON 1**–**ON 3** have been reported before.[6]
[b] Modified procedure: a converted order in deprotection and postsynthetic functionalization was employed, first removal of methyl protecting groups and afterward Stille coupling was carried out on bead.

Furthermore, we applied an initial experiment on Stille derivatization by employing TC chemistry, a protecting group strategy newly developed by Caruthers et al.[17] Similarly to TBDMS strategy, TC protecting group chemistry is based on use of 5′-*O*-dimethoxytrityl (DMTr), β-cyanoethyl for the phosphate and standard nucleobase protection as well as cpg as solid support allowing an almost identical setup for automated RNA synthesis. Instead of TBDMS, TC is utilized as 2′-*O* protecting group permitting a single-step basic deprotection using ethylenediamine under anhydrous conditions.[17] To the best of our knowledge, we pioneered the application of TC strategy in synthesis of modified RNA.[6] As described before, Stille cross-coupling was found to be fully compatible and **ON 2** had been completely converted to desired furyl-modified RNA in excellent yield and very high purity.[6] Formation of breakdown sequences was in case of TC protected RNA greatly diminished indicating high coupling efficiency. Thus, higher quantity of desired 12-*mer* could be isolated in comparison to TBDMS chemistry (compare to AE HPLC chromatograms in Fig. S1, Supplementary data). Besides integration of the chromatograms, this observation was confirmed in isolated amounts of modified RNA, too. Nevertheless, this is not due to applicability of Stille cross-coupling but rather attributed to differences in automated RNA synthesis, deprotection and isolation steps for TBDMS or for TC chemistry; as can be seen in RP HPLC chromatograms (Fig. S1, Supplementary data), transformation to furyl-coupled product had been over 90% for both strategies.

However, our excellent experiences with ACE chemistry, especially in combination with post-synthetic Sonogashira cross-couplings,[24] led us to test ACE protected RNA, immobilized on polystyrene support also for Stille cross-couplings.[6] Generally, ACE strategy provides a completely different approach requiring alteration of instrumentation since not acid-labile 5′-DMTr but fluoride-labile 5′-BzH [bis(trimethylsilyloxy)benzhydryloxysilyl] is used in combination with an acid-labile 2′-*O* orthoester. As consequence, no fluoride-sensitive cpg can be used as solid phase material but polystyrene support must be employed. For the same reason, cyanoethyl, which might be cleaved by fluoride, too, is substituted by methyl as phosphate protecting group.

Conform to studies with Sonogashira cross-couplings; high-yielded modified RNA (**ON 3**, Table 1) could be isolated. Coupling efficiency in automated oligonucleotide synthesis is very high for ACE chemistry (yields are described[14] to be so excellent that the only disadvantage, the lack of trityl monitor, is of no consequence). Preparation of ACE protected modified phosphoramidites, such as iodo nucleotides gives high yields and is very convenient. Particularly, no 2′, 3′-regioisomer mixtures being often observed in TBDMS chemistry form during the 2′-*O* blocking step. Moreover, the additional work-up procedure is less time-consuming, and being more practical in respect to handling under conditions not necessarily sterile, ACE strategy emerged as the most advantageous approach in our hands so far.

Nevertheless, poly uridine 12-*mer* oligonucleotide synthesized via ACE chemistry (**ON 3**) was separated from shorter failure sequences on anion exchange HPLC, desalted on Sephadex G25 material, and analyzed by mass spectrometry providing a surprising result. Crude **ON 3** showed besides desired mass an unexpected pattern with a series of additional +14 signals (see Fig. 1A).

An analytical sample of **ON 3** to RP HPLC also manifested the presence of several compounds (compare to Fig. 1B).

We supposed there might be a connection to the methyl group that is used as phosphate protecting group in ACE chemistry. We excluded incomplete methyl group removal after synthesis and derivatization since this would have been detected on anion exchange HPLC. Thus, methyl group transfer from the oligonucleotide backbone to the base residue seems to be due to conditions of Stille cross-couplings resulting in methylated uridine species.

In fact, methyl as phosphate protecting group provides a triester phosphate carrying two sugar residues and one methyl group, and thus resembles the alkylating agent TMP (trimethylphosphate). In various studies, conversion with TMP particularly under aqueous conditions turned out to be rather efficient in methylation of *N*1/*N*3 pyrimidines[27,28] and *N*1/*N*3 *N*7/*N*9 purines[27,29] as well as for *N*3 pyrimidine and *N*1/*N*6 purine ribo- and deoxyribonucleosides[30–33] and also on nucleotides[33], respectively. Since for methylation with TMP only one methyl group is offered and dimethylhydrogenphosphate forms,[30] the methyl protected phosphate backbone could be a mimetic and also cause methylation.

Furthermore, methylation of thymine residues during oligonucleotide synthesis was already earlier reported by Jones.[34] The authors claim that thymine residues are subjected to methylation at *N*3 via internucleotide methyl phosphotriester linkages. They performed model reactions with thymidine monomers, dimers, and hexamers in the presence of bases, such as DBU (1,8-diazabicyclo [5.4.0]undec-7-ene) or TEA (triethylamine) observing N-methylation and concluding that thymidine alkylation is a potential side reaction during oligonucleotide synthesis and deprotection steps. Actually, they found these impurities in small amounts indicating methylation processes. Nevertheless, they note that the extent of alkylation can be minimized by avoiding strong bases and concede that the small amount of methylated material might have no detectable effect for most applications.[34]

This might clarify why Scaringe et al.[14] claim in their original presentation about ACE strategy that no side-products could be detected supporting this by anion exchange HPLC chromatograms but without mass spectra. However, Jones et al.[34] observed the methylation side reaction via RP HPLC for DNA standard synthesis

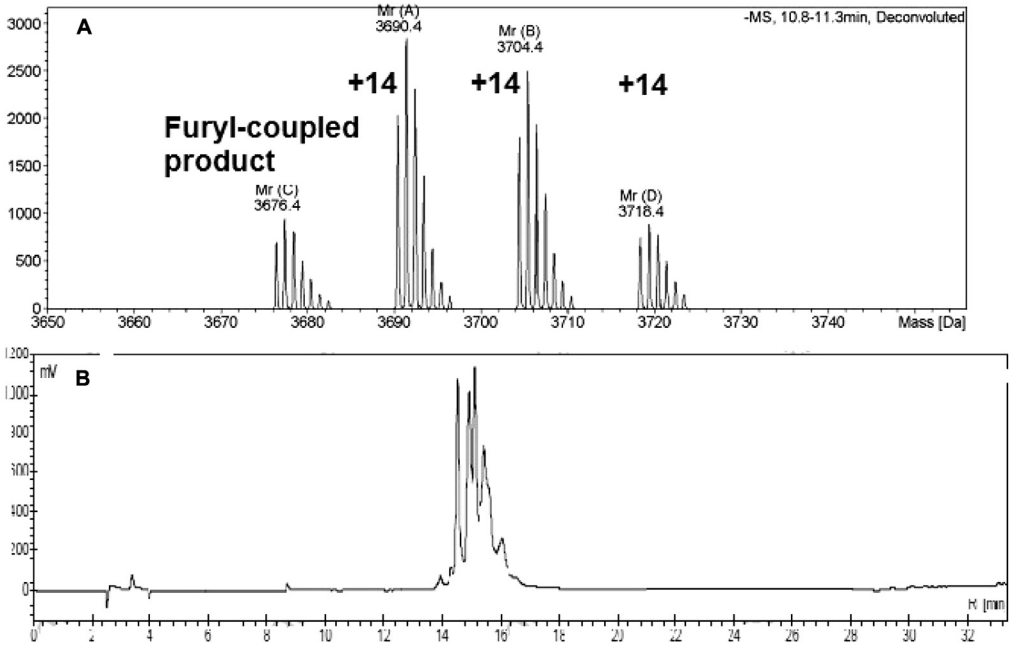


Fig. 1. ESI(−)-mass spectrum (A) and RP HPLC chromatogram (B) of poly uridine 12-*mer* RNA **ON 3** synthesized via ACE strategy and postsynthetically furyl-labeled via Stille cross-coupling: besides desired product also methylated species (+14) could be detected.

and deprotection conditions (if methyl was used as phosphate protecting group). Even in our hands we could detect methylated uridines on RP HPLC but not on anion exchange columns. Although no base is used in Stille cross-coupling, same effect of methylation (such as described by Jones et al.[34]) occurred in high degree here—RP HPLC and mass spectra showed not just traces of methylated material but approximately identical quantities for each of the four species (Fig. 1A and B).

2.2. Studies of methylation processes on RNA caused by Stille coupling

To further investigate this phenomenon, we first proved the presence of methylated uridine nucleosides. For this purpose, enzymatic digestion of the oligonucleotide was performed to allow subsequent analysis of free nucleosides. Moreover, the second objective involved test reactions on monomers based on conditions of Stille cross-coupling on RNA—therefore, high excess of reagents and addition of TMP were applied to mimic the protected phosphate backbone in RNA.

RNA oligonucleotides were degraded to free nucleosides by enzymatic digestion with endonuclease P1 followed by alkaline phosphatase for dephosphorylation. This was performed with poly uridine 12-*mer* **ON 3** that had been furyl-modified via Stille cross-coupling and being characterized as mixture of desired product and several species with additional +14 masses (compare to Fig. 1). As control experiment, completely unmodified 12-*mer* poly uridine **ON 4** was also subjected to enzymatic digestion. Analysis of digested oligonucleotides was performed via analytical RP HPLC. We supposed that base composition analysis of furyl-modified poly uridine 12-*mer* **ON 3** would reveal not only uridine **1** and 5-furyl uridine **3** but additionally *N*-methyl uridine **2** and 5-furyl-*N*-methyl uridine **4** (Fig. 2).

Signals from digested RNA, which was directly subjected to RP HPLC, could be assigned via comparison to reference chromatograms of monomer compounds **1**–**4**; and additionally, peaks were collected and confirmed by mass spectrometry analysis. In fact, digestion of unmodified 12-*mer* **ON 4** as control experiment showed the expected single peak of natural uridine (not shown); and for the examined furyl-modified 12-*mer* **ON 3** predicted uridine derivatives **1-4** were found, and thus, formation of methylated species was proven (see Fig. 2).

After enzymatic digestion, all four uridine species were detected indicating that no complete methylation of all 5-furyl uridine building blocks occurred, but rather a kind of statistic distribution for methylation process seems to happen. However, it is striking that probability of methylation for 5-furyl uridine seems to be highly increased compared to uridine (integral ratio in HPLC chromatogram: 5-furyl *N*-methyl uridine **4** to 5-furyl uridine **3** one order of magnitude higher than *N*-methyl uridine **2** to uridine **1**, compare to Fig. 2). This observation could be confirmed in studies concerning monomer tests that are described below and is probably due to higher reactivity of 5-furyl uridine towards electrophiles (compare to Section 2.3).

Monomer model reactions that were intended to simulate the setting for Stille cross-coupling on RNA, were carried out with uridine **1**, 5-iodo uridine **5**, or a mixture of both, respectively. The nucleosides were treated with TMP mimicking the methyl phosphate backbone under different setups involving Stille coupling conditions (Scheme 1, and compare to Table 2).

For that purpose, we monitored all test reactions by analytical RP HPLC.

First experiments using 1 equiv TMP and stirring at room temperature or at 60 °C showed that no transformation of uridine **1** (Table 2, exp. no. 1, 2, 5, 6, and 9) could be achieved independently of adding organotributylstannane or increasing the catalyst/ligand loading.

Model reactions on 5-iodo uridine **5** (exp. no. 3, 4, 7, 8, and 10) revealed that addition of tributylfuryl stannane is obligatory to mediate the methylation side reaction. That indicates that methylation cannot be explained by the only presence of catalyst and ligand even if palladium and ligand complexing the nucleoside might support the process. Particular conditions (exp. no. 3, 7, and 10) led to complete conversion of 5-iodo uridine **5** to 5-furyl uridine **3**, and methylation resulting in *N*-methyl 5-furyl uridine **4** could be detected. Besides analysis by analytical RP HPLC, one testing (exp. no. 7) was additionally worked-up and purified via column chromatography permitting not only mass spectrometry

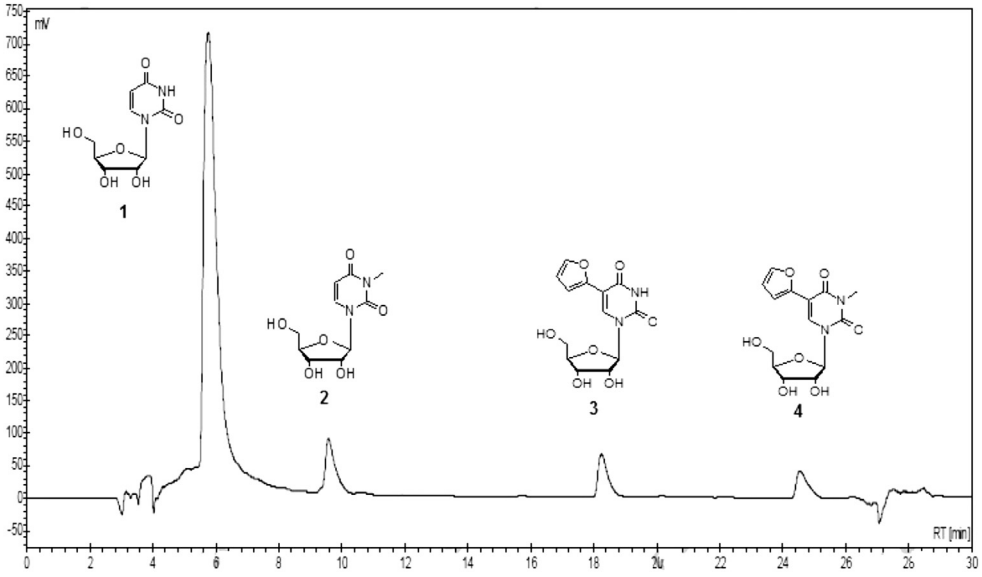
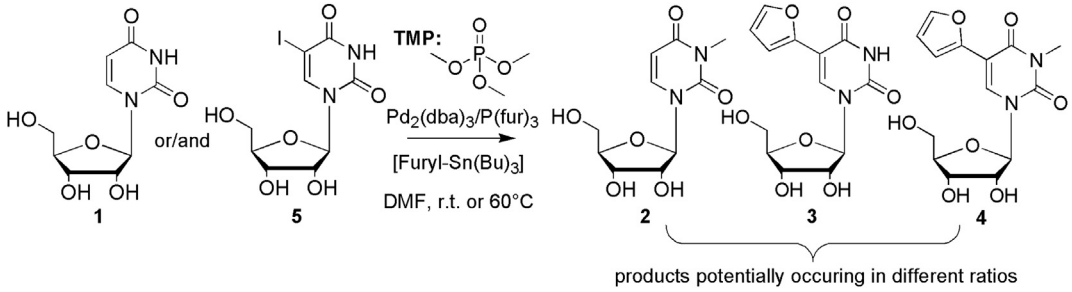


Fig. 2. RP HPLC chromatogram of furyl-modified uridine 12-*mer* **ON 3** (synthesized via ACE chemistry) after enzymatic digestion: monomeric uridine derivatives **1**–**4** were monitored.



Scheme 1. Monomer test reactions to mimic Stille cross-coupling under RNA conditions for examination of N-methylation (particular settings are given in Table 2).

but also NMR characterization, which as well identified both compounds as **3** and **4**. Conform to relations determined via integration of HPLC signals (compare Table 2, exp. no. 7), 37.5% 5-furyl uridine **3** and 62.5% *N*-methyl 5-furyl uridine **4** were isolated as pure compounds.

We further investigated the role of the catalytic cycle in regard to the fact that uridine alone seems not to get methylated in the test reactions (so far, exclusive methylation of furyl uridine **3** emerged what might be due to electronic and thus reactivity reasons; compare to results of oligonucleotide digestion, in which the amount of methylated uridine **2** formed in lesser extent than methylated furyl uridine **4**). The next experiment (exp. no. 11) consisted of employing both nucleosides uridine **1** and 5-iodo uridine **5** as they are coexisting in the oligonucleotide. Finally, considering the much higher reaction rate of intrastrand reactions and motivated by the setup used by Jones et al.[34] who increased concentrations of TMP for reaction on thymidine by reason that this might be a better model for the intrastrand reaction likely taking place on oligonucleotides, 10 equiv excess of TMP was used here. However, employing a mixture of **1** and **5** in the same setup (exp. no. 13) afforded approximately 80% methylated furyl compound **4**, and 20% **3**, while no starting material **5** was left. Moreover, besides of remaining uridine **1**, material identified as *N*-methyl uridine **2**, could be monitored indicating methylation of uridine **1**, too, in approximately 30% (Fig. 3). All peaks were assigned via retention time of reference compounds, and additionally collected and analyzed by mass spectrometry.

2.3. Mechanistic considerations

All our results indicate that methylated uridine species **2** and **4** formed under particular conditions. Interestingly, the extent of methylation was found to be rather different for unmodified uridine (methylation degree in **ON 3** approximately 8%, in monomer test, exp. no. 13, less than 30%) compared to 5-furyl uridine (methylation degree in **ON 3** almost 50%, in monomer test, exp. no. 13, approximately 80%). This difference in reactivity towards methylation might be due to the fact that nucleophilic attack of *N*3 of the uridine derivative is favored if the acidity of *N*3 is higher. In fact, the calculated pK_a value for uridine is 9.4±0.1 while it is decreased to 8.9±0.1 for 5-furyl uridine (calculated by ACD Persepta 1997–2012). This is in full agreement to the electron-withdrawing character of the furyl substituent stabilizing negative charge that results from deprotonation. Furthermore, we assume that the large excess of palladium catalyst and eventually ligand might lead to complexation of the nucleoside providing further stabilization of the deprotonated, nucleophilic uridine derivative. Particularly coordination of the metal between carbonyl in 6-position and a further oxygen, such as 1-*O* of furyl in compound **3**, forms a six-membered complex, which is energetically favored and stable. These effects based on the furyl substituent in **3** do not exist for unmodified uridine **1**, which is not surprisingly less reactive.

Additionally, we observed that a single uridine alone has not been methylated under Stille conditions but presence of 5-iodo uridine was required to initiate the catalytic cycle. Control tests

Table 2
Monomer test reactions to investigate methylation process mimicking conditions of Stille cross-coupling on RNA by addition of TMP[a]

Exp. no.	Start. mat.	Fur-Sn(Bu)$_3$	TMP	Result
1[b]	U (**1**)	+	1 equiv	No transformation
2[b]	U (**1**)	—	1 equiv	No transformation
3[b]	5-IU (**5**)	+	1 equiv	Traces of Me-Fur-U (**4**)
4[b]	5-IU (**5**)	—	1 equiv	No transformation
5[c]	U (**1**)	+	1 equiv	No transformation
6[c]	U (**1**)	—	1 equiv	No transformation
7[c]	5-IU (**5**)	+	1 equiv	35% Fur-U (**3**) 65% Me-Fur-U (**4**)
8[c]	5-IU (**5**)	—	1 equiv	No transformation
9[c,d]	U (**5**)	+	1 equiv	No transformation
10[c,d]	5-IU (**5**)	+	1 equiv	2 min: >90% Fur-U (**3**); 20 h: 20% Me-Fur-U (**4**)
11[c]	U (**1**)/5-IU (**5**)	+	1 equiv	15 h: 95% Fur-U (**3**), 5% Me-Fur-U (**4**), 100% U (**1**), 0% Me-U (**2**);
12[c]	U (**1**)	+	10 equiv	No transformation
13[c]	U (**1**)/5-IU (**5**)	+	10 equiv	18 h: 20% Fur-U (**3**) 80% Me-Fur-U (**4**); >70% U (**1**) <30% Me-U (**2**);

[a] Setup under particular conditions was based on standard Stille cross-couplings; reaction times were between 13 h and 24 h; analytical samples were quenched with water/acetonitrile, diluted, and subjected to analytical RP HPLC; ratios given in this table were determined via peak integration.
[b] Reaction was carried out at room temperature.
[c] Reaction was carried out at 60 °C.
[d] Increased loading of 1.2 equiv $Pd_2(dba)_3$ as catalyst and 2.6 equiv P(furyl)$_3$ as ligand were used to simulate high excess used for Stille cross-couplings on RNA.

was detected in analytical RP HPLC and additionally assigned by mass spectrometry.

Hence, results so far indicate involvement of tributyltin iodide to mediate methylation process as mechanistic explanation, but need to be further investigated to provide definite prove.

2.4. Remarks and further studies on ACE protected RNA

It must be noted that the foregoing considerations about methylation processes only concerned methyl phosphate protected RNA containing uridine. The effect and very clear indication of significant amounts of several methylated species were due to use of a poly uridine sequence, such as **ON 3**; and additionally, due to presence of 5-furyl uridine being more prone to deprotonation and nucleophilic attack towards electrophiles. In comparison, for poly adenosine, none, and for mixed sequences containing merely few uridines and 2-furyl adenosine (providing no NH in the ring system) instead of 5-furyl uridine as modified building block, only traces of methylated side product were observed[6] in high-resolution mass spectra if prepared via ACE chemistry. However, it was proven and further elucidated within this work that ACE chemistry using methyl protection on phosphorus provides a significant drawback if Stille cross-coupling is applied on uridine-rich sequences.

Moreover, we aimed to check a further setup to prevent the problem of methylation occurring on methyl phosphate protected RNA. Conditions of Stille coupling obviously promote methyl group transfer, and hence we planned to convert the order of Stille derivatization and methyl group removal to carry out Stille coupling

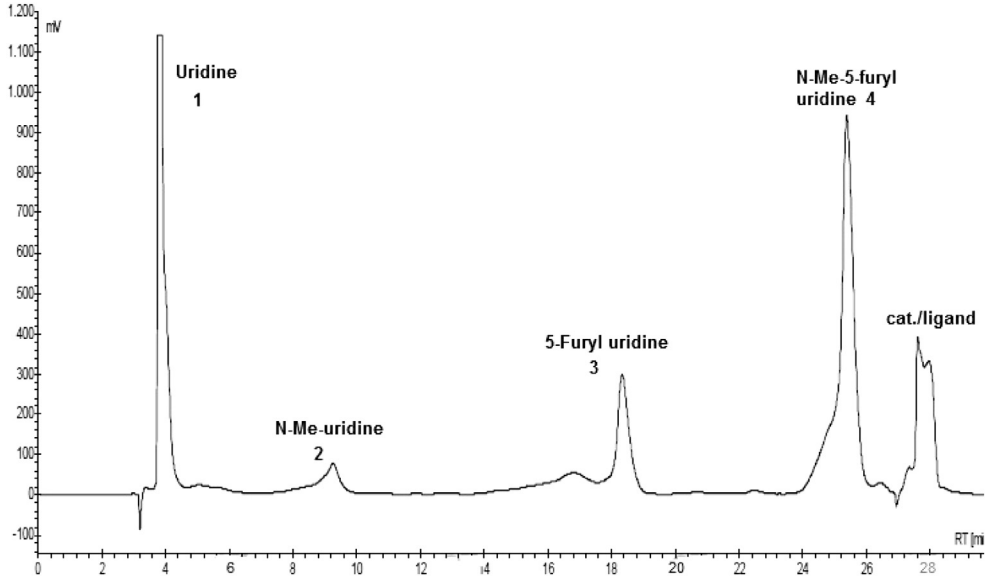


Fig. 3. RP HPLC chromatogram: methylation test on uridine **1** and 5-iodo uridine **5** under Stille cross-coupling conditions of RNA and addition of TMP mimicking the protected phosphate backbone (compare Table 2, exp. no. 13).

verifying this finding were carried out. Thus, we planned to check, whether an intermediate or rather side-product, such as tributyltin iodide that forms during catalytic cycle could play a role in mechanism of methyl group transfer. For that purpose, several experiments utilizing tributyltin iodide instead of tributylorganostannane and with or without catalyst/ligand were performed with uridine, 5-iodo uridine, a mixture of both or 5-furyl uridine. Particularly, test reactions with 5-furyl uridine (shown to be most reactive towards methylation due to electronic reasons, compare to results above) treated with $Pd_2(dba)_3$, P(furyl)$_3$, TMP, and $SnBu_3I$ revealed an outcome fitting well to our hypothesis. *N*-Me-5-furyl uridine **4** on RNA already lacking methyl groups. Since methyl group removal is a step employed independently from basic deprotection (that removes base protecting groups and releases RNA from solid support), methyl groups were removed prior to Stille coupling, which was subsequently performed on RNA with deprotected phosphate backbone but still bound on solid support, and finally the oligonucleotide **ON 5** was subjected to basic deprotection. Unfortunately, no intact **ON 5** could be detected by HPLC and mass spectrometry but only truncated sequences could be isolated.

Thus, Stille cross-coupling seems to be not compatible with RNA being deprotected on the phosphate backbone.

3. Conclusions

In conclusion, we studied the impact of selecting an appropriate protecting group strategy qualified for postsynthetic modification on RNA with respect to product formation, emerging side reactions and yields. Observed side-products arising on methyl phosphate protected poly uridine RNA under Stille cross-coupling conditions proved to be *N*-methylated material caused by methyl group transfer from the methyl protected phosphatetriester backbone. We investigated this phenomenon by enzymatic degradation of RNA, and subsequently identified and characterized resulting monomer building blocks. We performed monomer model reactions with TMP mimicking Stille conditions on RNA, which elucidated the methylation processes and role of methyl instead of cyanoethyl as phosphate protecting group in ACE chemistry. We discovered and explained that presence of Stille coupling conditions considerably increases the methylation activity.

Overall, we could demonstrate that TBDMS-protected RNA and newly developed TC chemistry are both equally qualified for postsynthetic derivatization by Stille coupling, while even higher coupling efficiency in automated oligonucleotide synthesis was achieved via TC strategy. In contrast, applicability of the otherwise very advantageous—since being mild and high yielding in RNA synthesis—ACE strategy is limited in postsynthetic Stille cross-coupling involving uridines, particularly carrying electron-withdrawing groups, due to methyl migration resulting in methylated side-products.

4. Experimental section

4.1. General information

All reagents were purchased from Aldrich or Alfa Aesar and were used as received.

CAUTION: Organo(tributyl)tin reagents and trimethylphosphate are hazardous compounds and can harm health via inhalation, skin contact and ingestion.

NMR spectra were recorded on Bruker AV instruments at 300 MHz and 300 K. Chemical shifts (δ) are reported in parts per million relative to the solvent signal. The fine structure of proton signals was specified with s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), dt (doublet of triplet), bs (broad singlet).

Thin layer chromatograms (TLC) were recorded on 60 F_{254} plates from Merck (thickness of layer 0.2 mm). Column chromatography was carried out on silica gel 60 (40–63 μm, Merck).

Reversed Phase (RP) HPLC was performed on a Jasco LC-2000Plus HPLC system equipped with a Jasco UV 2075Plus detector (detection at 254 nm).

ESI-Mass spectrometry was performed on a Fisons instrument equipped with a VG platform II with quadrupol analyser or on a Nano-ESI Mariner Biospectrometry Workstation from PerSeptive Biosystems.

High-resolution mass spectra (HRMS) were measured on a MALDI Orbitrap LTQ XL instrument (Thermo Fisher).

RNAs **ON 1**–**ON 4** were synthesized via ACE, TBDMS, or TC strategy, postsynthetically modified, deprotected, purified, and characterized as described before.[6] **ON 5** was subjected to same procedures but with the difference of converted order in methyl deprotection and postsynthetic modification: methyl groups were removed directly after RNA synthesis, subsequently Stille coupling was performed, and finally basic deprotection and cleavage from solid support was carried out.

N-methyl uridine **2** as reference compound has been prepared in analogy to methylation of thymidine reported by Jones et al.[34] Mass spectrometry after separation on RP HPLC showed a signal in full agreement with literature.[35] Analytical reversed phase HPLC after enzyme digestion and to monitor monomer model reactions was performed on an analytical (250×4.6 mm) Phenomenex C12 column [gradient: A: 1 M TEAA (triethylammonium acetate) buffer, pH 6.5; B: Millipore water, C: acetonitrile; constant 10% A, 0–20 min: 3–15% C and 20–21 min: 15–18% C, 21–23 min: 18% C, 23–25 min: 18–80% C, 25–30 min: 80–3% C; 1 mL/min flow].

4.2. General procedure for enzymatic digestion[21]

Typically 4 nmol RNA was dissolved in 100 μL acetate buffer (30 mM sodium acetate, pH adjustment to 5.4 by acetic acid) and 25 μL (300 units/mL) Nuclease P1 (from *Penicillium citrinum*, Sigma–Aldrich) was added. The mixture was incubated for 2 h at 37 °C and afterward 40 μL dephosphorylation buffer (0.5 M Tris–HCl and 1 mM EDTA, pH adjustment to 8.5 by HCl) and 1 μL (10 units) alkaline phosphatase (from calf intestine, Sigma–Aldrich) were added. After incubation for another 2 h at 37 °C, the mixture was directly injected to analytical RP HPLC.

4.3. General procedure for methylation test reactions on monomers

Model reactions on uridine **1** and 5-iodo uridine **5** were based on common handling and proceeding for Stille cross-couplings on monomers and RNA with tributylfuryl stannane as described before (typically 25 mg or 50 mg nucleoside, 0.054 equiv $Pd_2(dba)_3$/0.12 equiv $P(fur)_3$, 1.1 equiv tributylfuryl stannane in 1 mL abs. DMF, 60 °C, 13–24 h)[6] with the difference that trimethylphosphate was added in all cases—particular conditions and changed parameters are presented in Table 2. Experiments were controlled by RP HPLC to monitor conversion products, and besides assignment of compounds **1**–**4** via comparison by retention times of reference chromatograms, peaks of all four compounds were collected and additionally analyzed by mass spectrometry.

The new compound *N*-methyl-5-furyl uridine (**4**) formed in several experiments, and was analyzed by mass spectrometry, but was supplementary worked-up, isolated, and purified in case of exp. no. 7 (Table 2).

*4.3.1. N-methyl-5-furyl uridine (**4**).* The reaction was carried out with 50 mg (0.135 mmol) 5-iodo uridine **5** under conditions described above. After stirring for 20 h, the concentrated reaction mixture was purified by column chromatography (eluent 9:1 DCM/MeOH) to yield 15 mg (0.048 mmol) to give 5-furyl uridine **3** (expected and common product in particular Stille cross-coupling, analytical data in full agreement to those already reported[6]) and 25 mg (0.077 mmol) *N*-methyl 5-furyl uridine **4** as off-white solid.

R_f=0.46 (9:1 DCM/MeOH);

^{1}H NMR: δ [ppm] (300 MHz, DMSO-d_6): 3.25 (3H, s, *N*-C*H*$_3$), 3.58–3.64 (1H, m, 5′-C*H*), 3.69–3.75 (1H, m, 5′-C*H*), 3.91–3.94 (1H, m, 4′-C*H*), 4.03 (1H, q, *J*=5.0 Hz, 3′-C*H*), 4.12 (1H, q, *J*=4.9 Hz, 2′-C*H*), 5.10 (1H, d, *J*=5.4 Hz, 3′-O*H*), 5.22 (1H, t, *J*=4.6 Hz, 5′-O*H*), 5.43 (1H, d, *J*=5.4 Hz, 2′-O*H*), 5.90 (1H, d, *J*=4.5 Hz, 1′-C*H*), 6.53 (1H, dd, *J*=1.8 and 3.3 Hz, furyl-*H*4″), 6.91 (1H, dd, *J*=3.1 Hz, *J*=0.7 Hz, furyl-*H*3″), 7.61 (1H, dd, *J*=1.8 Hz, *J*=0.8 Hz, furyl-*H*5″), 8.53 (1H, s, 6-C*H*);

^{13}C NMR: δ [ppm] (75 MHz, DMSO-d_6): 27.61 (*N*-C*H*$_3$), 60.23 (5′-C*H*$_2$), 69.43 (3′-C*H*), 74.12 (2′-C*H*), 84.70 (4′-C*H*), 89.39 (1′-C*H*), 104.65 (5-*C*-furyl), 107.87 (furyl-*C*3″), 111.48 (furyl-*C*4″), 134.34 (6-C*H*), 141.53 (furyl-*C*5″), 146.48 (furyl-*C*2″), 149.79 (2-*C*O), 159.19 (4-*C*O);

ESI(−)-MS: calcd: 324.10, found (m/z): 323.1 $[M-H]^-$, 359.2 $[M+Cl]^-$;

HRMS (M+Na): calcd 347.08497, found: 347.08525.

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Supplementary data

Supplementary data related with this article include copies of mass spectra and HPLC chromatograms of **ON 1** and **ON 2**, as well as copies of NMR spectra and HRMS spectrum of novel compound **4**, and mass spectrum of **2**. http://dx.doi.org/10.1016/j.tet.2013.11.061.

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